Eccentric cleavage products of beta carotene: biologically active?

A Senior Honors Thesis

Presented in Partial Fulfillment of the Requirements for graduation
with research distinction in Chemistry in the undergraduate
colleges of The Ohio State University

by

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List of Abbreviations

DIBAL- diisobutyl aluminum hydride
h- hour(s)
HPLC- high performance liquid chromatography
LAH- lithium aluminum hydride
min- minute(s)
MS- mass spectrometry
NMR- nuclear magnetic resonance spectroscopy
rf- retention factor
rb- round-bottomed
rt- room temperature
THF- tetrahydrofuran
UV/Vis- ultra-violet/visible

Introduction

β-Carotene Metabolism

The most important role of β-carotene has long been thought to be the metabolic cleavage at the central double bond to produce two molecules of vitamin A (shown in Fig. 1). β-carotene-15,15'-oxygenase (BCO1) has been identified as the enzyme responsible for this cleavage. Retinal has been shown to be the exclusive cleavage product of this enzyme.[1]

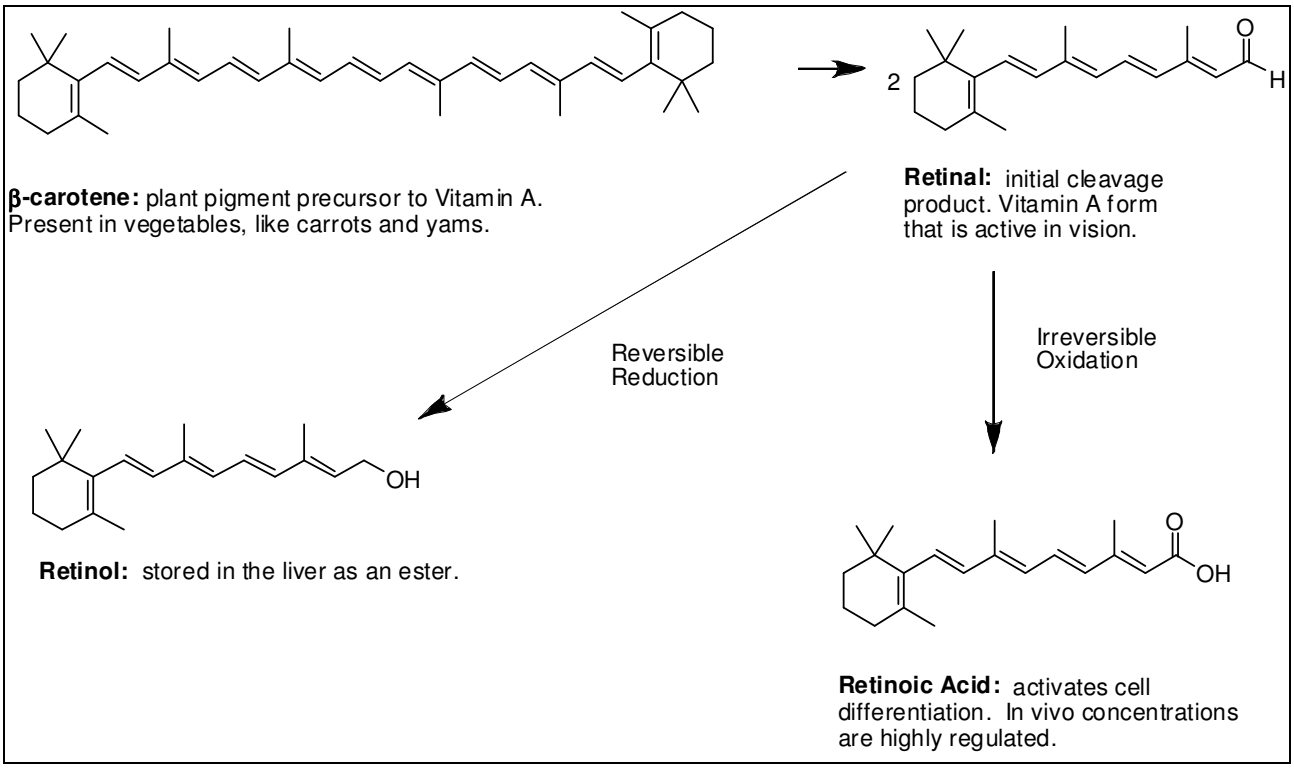

Figure 1. A scheme of the general metabolism of β-carotene.

A second enzyme, β-carotene oxygenase 2 (BCO2), was isolated and characterized in 2001 from mice. This enzyme catalyzes the oxidative cleavage of the 9'-10' double bond of β-carotene. The human gene for this enzyme's analogue was also found through cDNA analysis.[2] These discoveries suggest that the presence of apo-carotenoids *in vivo* might be due to metabolic processes and not just chemical degradation of β-carotene. This might mean that β-carotene might have other biological functions other than its vitamin A functions via its eccentric cleavage products.

A 2007 study found that ^{14}C-labeled β-apo-8`-carotenal was being excreted from a healthy man 3 days after a dose of labeled β-carotene. However, the apo-carotenal product was identified strictly based on a matched retention factor with a standard using high performance liquid chromatography (HPLC).[3] This investigation seems to provide verification that humans can cleave β-carotene "eccentrically"—at bonds other than the 15,15' central double bond. More studies are needed to quantitate the occurrence of apo-carotenoids in humans, to explore the enzymology that leads to their formation, and to explore the biological function of eccentric cleavage products.

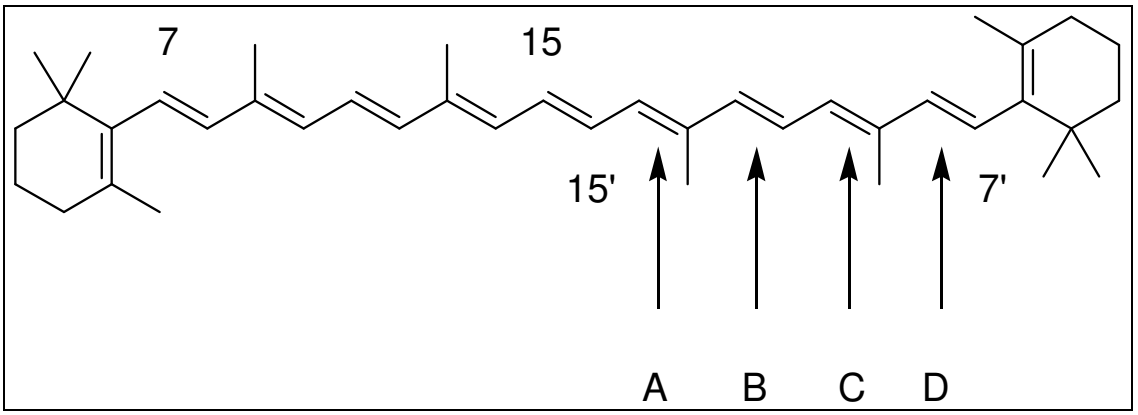

Figure 2. An illustration of the potential eccentric cleavage sites in β-carotene. The numbers above and below illustrate the numbering scheme used to name apo-carotenoids. The carbons on the polyene backbone are counted up from 7 to 15, then down from 15' to 7'.

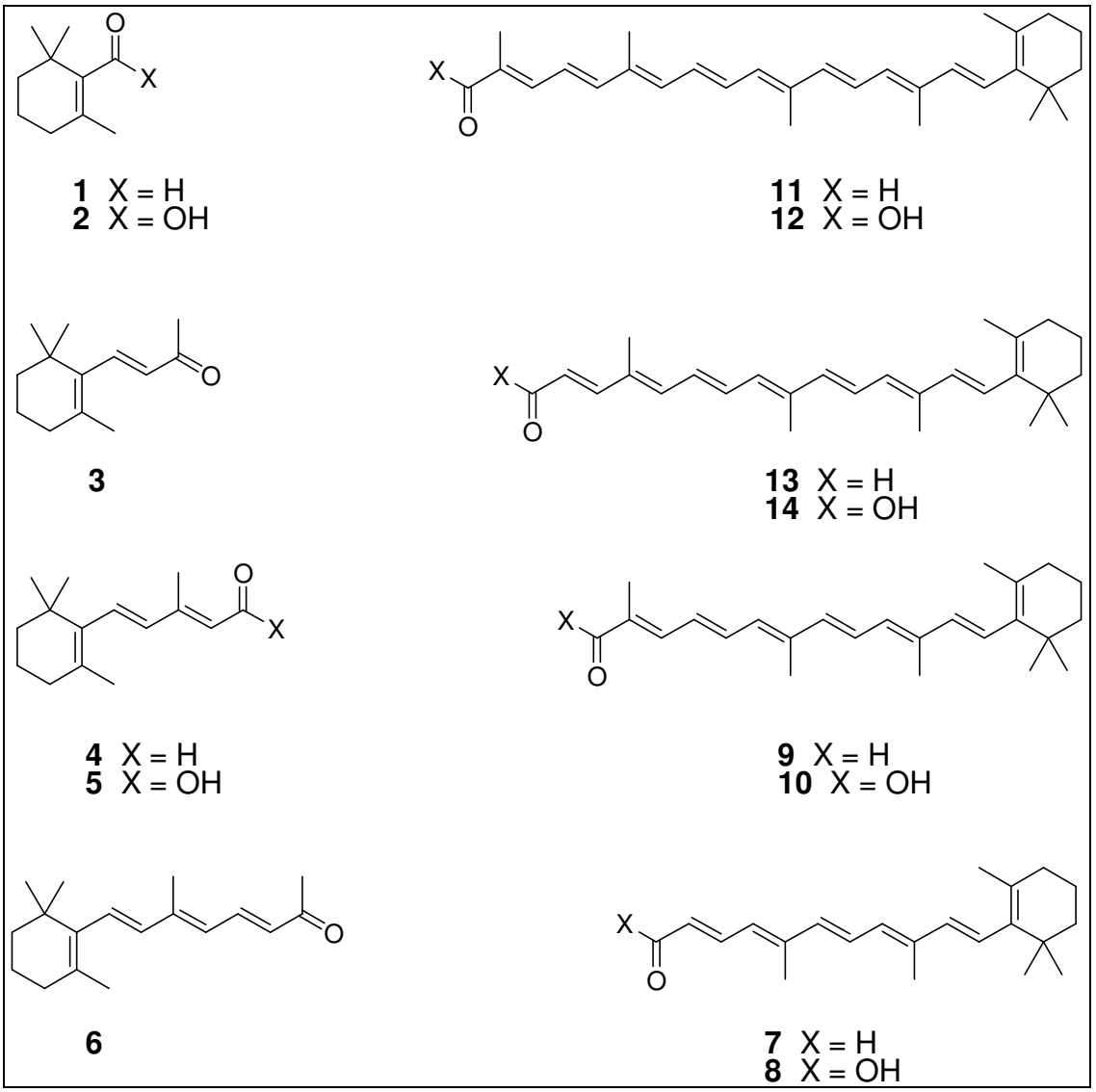

Figure 3. An illustration of all the possible putative eccentric β-carotene metabolites. These numbers will refer to the compounds throughout the text.

We seek to explore the metabolism and function of β-carotene's eccentric cleavage products. We are synthesizing and characterizing all of the possible aldehyde and acid metabolites of β-carotene (See Figure 3) for use as analytical standards and for direct assay of their biological activity. Multi-step syntheses were employed to synthesize many of the molecules. Nuclear magnetic resonance spectroscopy, UV-visible spectrophotometry, high performance liquid chromatography, and mass spectrometry are being used for characterization. The laboratory of Dr. Earl Harrison (Dept. of Nutrition) is currently studying the interaction of the eccentric cleavage products with the nuclear retinoic acid receptors (RARs), which are responsible for cell differentiation and aspects of the immune response by binding to the central cleavage product retinoic acid. The interaction of these molecules with other nuclear receptors will be analyzed in the future. The samples will also be used as standards to quantify the concentrations of eccentric cleavage products in biological samples. The synthesis of the molecules prepared to date will be described as well as aspects of the limited biological activity data gathered to date.

Lycopene is an intermediate in the biosynthesis of β-carotene and is present as a pigment in many fruits and vegetables in the diet. Lycopene is not considered an essential nutrient, but considerable interest has revolved around its use as a chemopreventive agent. Not surprisingly, apo-lycopenals have been found as metabolites of lycopene in rats.[4] Moreover, both BCO1 and BCO2 have demonstrated slow cleavage of lycopene.[3,5] Ozonolysis of lycopene and subsequent chromatography is being explored as a potential method to isolate putative lycopene metabolites so that their biological function can also be explored.

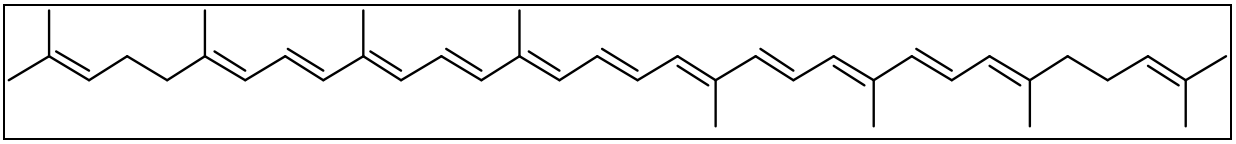

Figure 4. Lycopene.

Discussion of Syntheses and Preparations

*Synthesis of β-apo-14`-carotenal (Compound **7**)*

Figure 5 summarizes the approaches taken to synthesize β-apo-14`-carotenal. Route 1 was initially used to synthesize **7** (Wadsworth-Emmons addition of triethylphosphonoacetate, reduction with LAH, flash chromatography to isolate the alcohol, immediate elution through MnO_2 column). Cardoso *et al*. used a similar approach to synthesize **7** in 1996.[6] They synthesized the methyl ester of β-apo-14`-carotenoic acid (instead of the ethyl ester), reduced this compound with 2 equivalents of DIBAL, and oxidized the alcohol to **7** by stirring with MnO_2 in THF. No yields were reported, but the synthesis was efficient enough to produce enough **7** to be used as a starting material for the synthesis of the longer-chain apo-carotenoids.

The major difference in our initial approach was the use of LAH instead of DIBAL as the reducing agent. LAH was chosen because its greater reactivity produces a more complete reduction to the alcohol in a shorter amount of time than DIBAL. LAH performed better in reductions of the β-apo-14`-carotenoate and the ethyl ester of β-ionylidene acetic acid (Compound **5**). The following observations were typical when comparing the two reducing agents:

1. Reaction of 3 equivalents of LAH with β-apo-14`-carotenoate in dry THF almost completely consumed the starting material in under ten minutes of reaction at room temperature (as checked by thin layer chromatography).
2. Reaction of 4 equivalents of DIBAL in CH_2Cl_2 with β-apo-14`-carotenoate still did not consume all of the starting material after 1.5 h of stirring at room temperature.

Moreover, the work-up of the DIBAL reaction is tedious and time-consuming. A 1 M aqueous solution of Rochelle's salt solution is added to the reaction mixture to break up the aluminum complexes formed. This aqueous layer is very difficult to separate from the CH_2Cl_2 layer because of emulsification between the two layers. Work-up of the LAH is greatly facilitated by a procedure that precipitates the aluminum reagent into a granular precipitate that is easily filtered off and washed.[7]

The use of DIBAL for the reduction of β-apo-14`-carotenoate might still be explored in the future. In the reaction described above, the β-apo-14`-carotenoate was not recovered. Recovery of the starting material might produce a more acceptable overall yield.

Both LAH and DIBAL produce two distinct aldehyde bands on a TLC plate after MnO_2 oxidation (rf = 0.38 and 0.46 in 4:1 hexanes:ethyl acetate) in an approximately 1:1 ratio. The lower band was the desired product, as its ^{1}H-NMR resonances matched the peaks listed by Cardoso *et al.* for β-apo-14`-carotenal.[6] ^{1}H-NMR spectra of these bands suggest that the double bond isomerization that produces the second band occurs at a site other than the terminal double bond. The major aldehyde doublets of both bands share an almost identical chemical shift (see Figures 1 and 2 in the appendix), a situation that would not arise if the local electronic environment were changed by cis-isomerization at the terminal C-C double bond. We hypothesize that this isomer is a result of some kind of complexation with the reagents' metal center. The higher aldehyde band was dissolved in ethanol and exposed to UV light (λ_{max} = 365

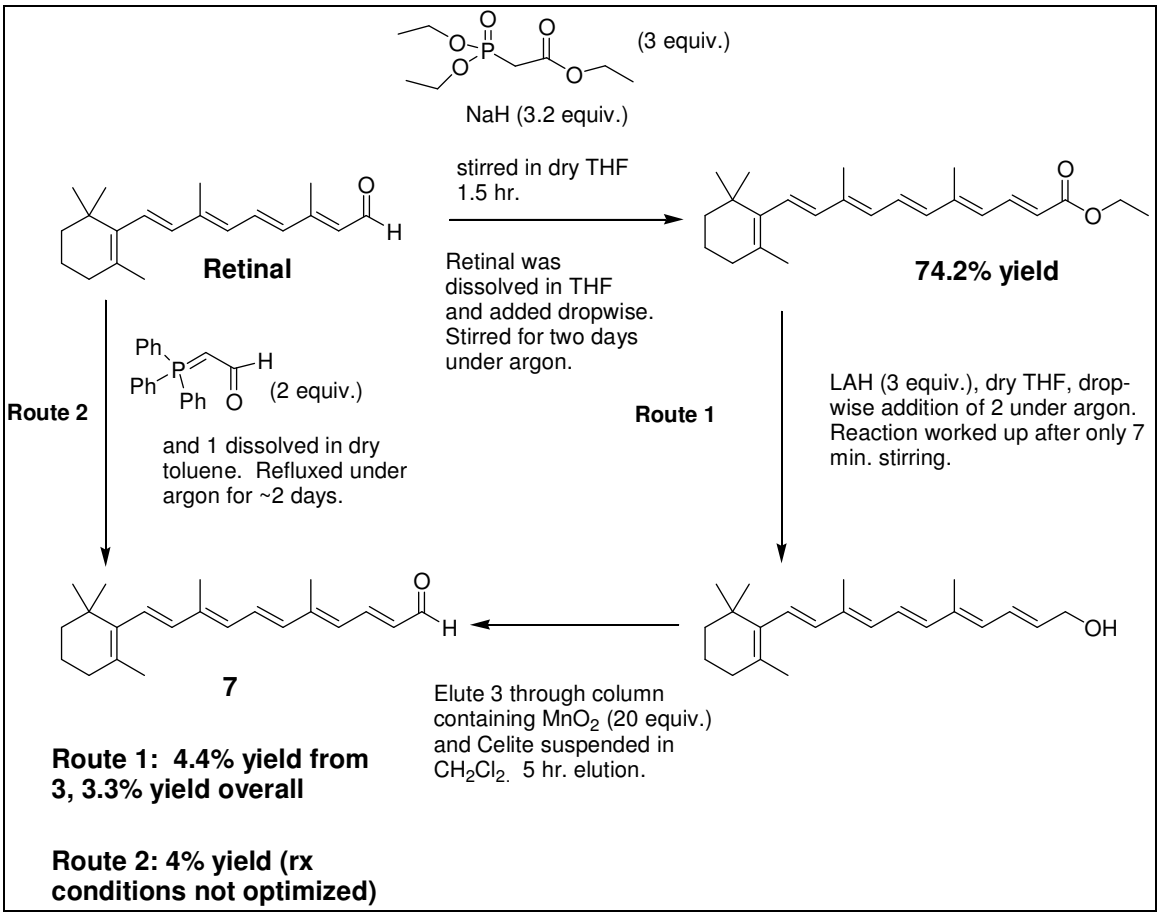

Figure 5. Summary of the synthetic routes towards β-apo-14`-carotenal

nm). TLC analysis after two hours of exposure suggested that the lower band became the predominant product, as a dark band appeared at rf = 0.38. Although no further analysis was done on this experiment, photoisomerization to the thermodynamically preferred product might be another method for increasing the yield of **7**.

Route 1 to compound **7** was deemed unsatisfactory after repeated attempts at optimizing reaction conditions. Too much product breakdown seemed to occur during the reduction step in the synthesis. The Wittig addition using (triphenylphosphoranylidene)acetaldehyde is now being optimized as a way to circumvent the problematic reduction step.

The initial attempt of the Wittig addition shown above already provides a greater overall yield of **7**. This initial attempt has revealed some problems associated with this synthesis:

1. Multiple additions of the Wittig reagent occur because each Wittig addition produces an aldehyde site active towards another addition. The presence of the presumed multiple adducts is observable through reverse-phase TLC and through HPLC (See Figure 3 in the Appendix).
2. Two purification steps are needed because
    a. Silica gel chromatography did not resolve retinal, β-apo-14`-carotenal, and other all-trans adducts.

b. Reverse phase chromatography did not effectively resolve cis-trans isomers.

The synthesis of β-apo-14`-carotenal needs to be optimized further because β-apo-14`-carotenal is the starting material for the synthesis of the longer apo-carotenoids. Moreover, it is the product of the cleavage site adjacent to the central double bond, so its structural similarity to vitamin A might yield some interesting biological properties. The Wittig reaction is currently still being pursued as a potential method for the efficient synthesis of this compound.

*Synthesis of β-apo-13-carotenone (Compound **6**)*

The synthesis and characterization of β-apo-13-carotenone is of particular interest because molecular modeling predicts a strong binding interaction between RARβ and **6**, maybe even better than retinoic acid. β-apo-13-carotenone is therefore a strong candidate as an agonist or antagonist of the activity of retinoic acid.

Figure 7 summarizes the multi-step reaction scheme used to synthesize **6**. Compound **6** was synthesized from β-ionylideneacetaldehyde (Compound **4**). The preparation of **4** used the same approach as Route 1 in the synthesis of β-apo-14'-carotenal (Compound **7**): Wadsworth-Emmons addition of triethylphosphonoacetate to β-ionone (Compound **3**), reduction with LAH, immediate elution through MnO_2 column. LAH is clearly the superior reagent to DIBAL for this reduction. The LAH reduction completely reduced the ester to the alcohol in 45 minutes without any apparent side product formation (as tracked by TLC), so the alcohol product was eluted through a MnO_2 column without flash column purification.

Our initial synthesis of **6** from **4** (Route A in Figure 7) used the still inactive acetonyltriphenylphosphonium chloride. NaH was used to deprotonate and activate the Wittig reagent. This synthesis was very inefficient (1.7% yield) for several reasons:

1. The reaction was repeatedly heated in different solvents with new portions of phosphonium salt and NaH (THF reflux for 2 days; benzene reflux overnight; toluene reflux for two days and stir at rt for two days). This may have caused product breakdown.
2. NaH and the phosphonium chloride were not very soluble in benzene or toluene. The heterogeneous mixture may not have effectively deprotonated the phosphonium salt.
3. These reactions were being tracked by TLC, and a different rf for **6** was expected.

Interestingly, **6** has a lower rf on silica gel TLC (0.35 in 4:1 hexanes:ethyl acetate) than both **3** and **4** (0.50 and 0.41 respectively). This is surprising because **6** has the same methyl ketone headgroup as **3** with a longer hydrocarbon chain. An rf of at least 0.5 was therefore expected to be observed for the product. The surprisingly low rf may be due to a propensity to undergo keto-enol tautomerization due to the stabilizing effect of the longer conjugated hydrocarbon chain.

The synthesis of **6** from **4** was repeated using Route B: reflux 1.5 equivalents of 1-triphenylphosphoranylidene-2-propanone with **4** overnight in dry toluene under argon. A similar

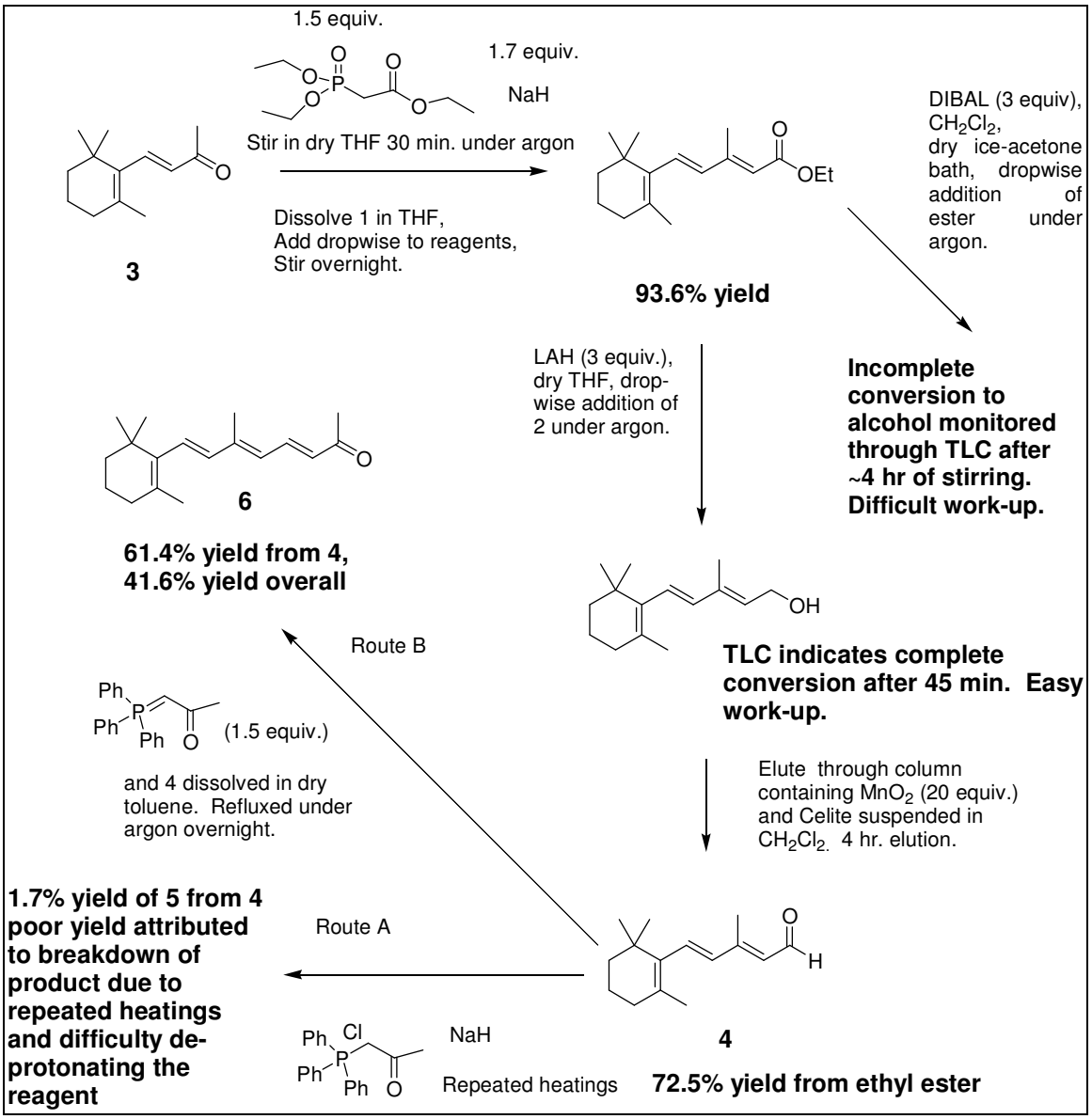

Figure 7. Summary of the routes attempted to synthesize β-apo-13-carotenone (Compound **6**)

synthesis, where 1.25 equivalents of phosphorane was refluxed with **4** in dry toluene under nitrogen for 48 h, was reported in 2006.[8] Our second synthesis was complicated because the product that had been synthesized through Route A, which was being used as a TLC standard, had completely decomposed into something else (rf = 0.79, NMR spectrum shown in Figure 4 in the Appendix) even though it had been stored neat in the freezer at -20º C for about a year. Interestingly, a portion of this first synthesis product, which had been stored in the freezer in ethanol, showed no signs of product degradation. Hereafter, **6** will be stored in ethanol.

Other Syntheses and Preparations

β-Cyclocitral (Compound **1**) and β-ionone (Compound **3**) were obtained through chromatographic purification of crude purchased products. β-apo-8'-carotenal (Compound **11**) was purchased. Other general synthetic methodologies are summarized in Figure 8.

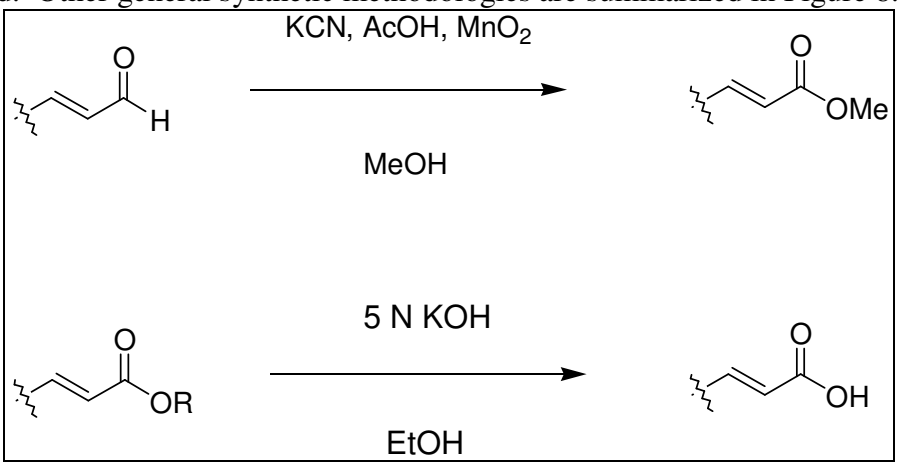

Figure 8. General scheme of cyanide catalyzed oxidations of allylic aldehydes and saponification of esters.

The cyanide-catalyzed oxidation of an allylic aldehyde to a methyl ester is a convenient oxidation method that avoids cis-trans isomerization at the olefinic linkage site.[9] This method has been applied to the esterification of β-apo-8'-carotenal, which was saponified to β-apo-8'-carotenoic acid (Compound **12**). These reaction conditions seemed too vigorous for the conversion of β-cyclocitral to β-cyclogeranic acid (Compound **2**) Saponifications have been used for the syntheses of β-apo-14'-carotenoic acid (Compound **8**) and β-ionylidene acetic acid (Compound **5**).

More Economical Production of Retinal

Retinal is the key starting material for the synthesis of longer chain apo-carotenoids but very expensive to purchase. The cost of this starting material was dramatically reduced by converting retinyl acetate into retinal through a saponification and subsequent oxidation to retinol. This reaction scheme is summarized in Figure 9.

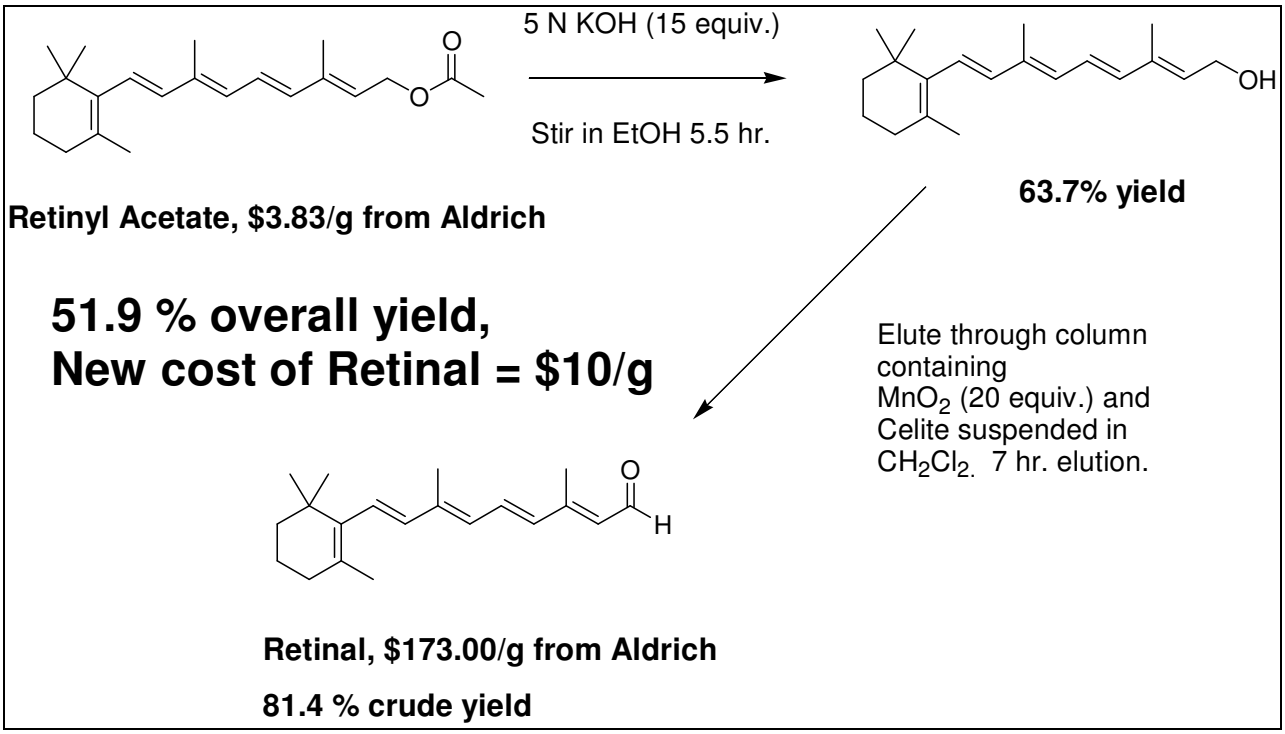

Figure 9. Summary of the conversion of retinyl acetate into retinal.

Development of the MnO_2 Oxidation Column

Activated MnO_2 is used as a reagent for partial oxidation of primary allylic alcohols to aldehydes. Solladie *et al.* report full conversion of retinol to crude retinal using an MnO_2 oxidation (5 equivalents of MnO_2 in dichloromethane, stirred for 2 hr).[10] Our initial reaction conditions were similar (10 equivalents of MnO_2 in dichloromethane, stir overnight under argon), but they never yielded a complete conversion to the aldehyde and often produced side products.

Solladie *et al.* used activated MnO_2 obtained through the Attenburrow procedure[11] for their retinol oxidation, while the reagent used in our reactions was purchased from Sigma-Aldrich. Fieser and Fieser note that commercially available activated MnO_2 is even more active than the Attenburrow oxide.[12] Therefore, water adsorbed to oxidatively active sites may have inactivated our MnO_2. Goldman reports storing activated MnO_2 under benzene.[13] The reagent used in the initial procedure was being stored in a desiccator at rt. This may not have been enough to prevent over-hydration of the MnO_2.

A study was carried out to optimize the use of MnO_2 for the transformation of retinol to retinal. The following reaction conditions were compared:

1. Elution through a column containing a suspension of diatomaceous earth (Celite) and 20 equiv. of oven-dried MnO_2 for 4 h.
2. Stirred retinol in a suspension of 20 equiv of oven-dried MnO_2 in CH_2Cl_2 under argon.
3. Stirred retinol in a suspension of 20 equiv. of non-oven-dried MnO_2 in CH_2Cl_2 under argon.
4. Stirred retinol in a suspension of 3 equiv. of $BaMnO_4$ in CH_2Cl_2 under argon.

The reactions were tracked through thin layer chromatography. TLC analysis showed almost complete conversion to retinal after four hours in the column oxidation. Crude retinal was isolated in a 62.4% yield. The stirring reactions were checked by TLC after 4.5 h. None of these reactions yielded a full conversion to retinal. The degree of conversion to retinal based on TLC analysis was ordered as follows: $2 > 3 > 4$. These reactions were left to stir for 3 days. TLC analysis indicated that they had essentially not moved any further towards completion than at the initial check after 4.5 h. The crude material of these three reactions was combined and purified through preparative TLC to yield retinal (34.6% yield).

The column oxidation provided the fastest and most complete conversion to retinal. All further allylic alcohol oxidations were carried out using the column oxidation procedure. Storage of the MnO_2 was moved from a desiccator to a 120° C oven to prevent hydration of the reagent.

Various Celite:MnO_2 ratios were used in the column oxidations (5:1, 7.5:1, 10:1). This ratio seems to have no effect on the efficiency of the column oxidation. Elution time has more of an effect on the conversion. Column elutions over 3.5 h have generally demonstrated essentially complete conversion to aldehyde.

Lycopene Ozonolysis

Ozonolytic cleavage might be a way to conveniently access putative metabolites of lycopene. Our goal is to reproducibly ozonize lycopene so that its putative biological cleavage products can be isolated through preparative HPLC. Initial small scale ozonolysis attempts have been analyzed using HPLC-MS by our collaborators in the Department of Food Science (Dr. Steven Schwartz). Their results indicate that the ozonolysis reaction is producing desired products. HPLC traces of the product mixture show sharp peaks that may contain the putative metabolites (see Figure 5 in the Appendix).

Lycopene isolation

Since lycopene is a very expensive starting material to purchase ($116.50/mg), a method of obtaining crude lycopene from tomato paste was developed. The method of Feruzzi et al. was used to isolate a tomato oleo resin from tomato paste.[14] We found crystallization of lycopene from this oleo resin to be inefficient, so we isolated crude lycopene through chromatography. The crude extract seemed to react with the silica gel. Reverse-phase chromatography was then used. A 45:45:10 MeOH:CH_3CN:CH_2Cl_2 mobile phase was used on reverse phase preparative plates because this mobile phase had been used to semi-preparatively purify lycopene through HPLC.[15] The band at the origin on these plates was found to contain lycopene and other long-chain lycopenoids through an HPLC-MS analysis conducted by our collaborators in the Department of Food Science. Reverse phase column chromatography was then used to isolate crude lycopene on a larger scale.

Ozonolysis

"Reductive ozonolysis," which directly produces carbonyl products and avoids ozonide intermediates, was used to ozonize the lycopene. Schwartz *et al*. discovered that amine oxides nucleophilically attack the carbonyl oxide intermediates of ozonolysis and that this intermediate subsequently fragments into a carbonyl product, an amine, and dioxygen.[16]

These ozonolysis reactions used *N*-methylmorpholine-*N*-oxide (NMMO) as the amine oxide. Each reaction included 3.5 equivalents of amine oxide per double bond in lycopene. Phosphate buffer (pH = 6) was added to the reaction mixture after addition of ozone to prevent base-promoted fragmentation.[16] The degree of ozonolysis was tracked qualitatively by observing the color of the solutions. The solutions became less and less colored as more ozone was added because bonds in conjugation were being cleaved.

Initially, the gas coming out of the ozone generator was bubbled straight into the reaction mixture. These reaction conditions proved hard to control, as the solutions immediately lost their color upon exposure to the gas.

Saturated solutions of ozone in CH_2Cl_2 were then created by bubbling the gas from the ozone generator into dichloromethane. Aliquots of this saturated solution were then transferred into the NMMO-lycopene dichloromethane solutions. These reactions introduced some level of

control and reproducibility. The solutions did not immediately lose their color upon contact with the saturated ozone solution, and the amount of saturated solution added could be controlled.

However, qualitative observations suggested that the ozone concentration in the saturated solution varied. Oftentimes, addition of the same aliquot of ozone solution to the same amount of lycopene produced a qualitatively different color. Nonetheless, some of these ozonolysis reactions were shown to produce the desired cleavage products.

A new, more standardized method is being developed for the addition of an ozone solution to a lycopene solution, where the concentration of the ozone solution is quantified. The molar absorptivity of ozone in water has been determined to be: $\varepsilon_{260} = 3292$ ($M^{-1}cm^{-1}$).[17] A UV/Vis spectrum of ozone in dichloromethane resulted in a λ_{max} of 260.5 nm, which shows that ozone has similar absorption behavior in dichloromethane and water. The following experiment shows the potential for variability in ozone concentration using the above procedure.

Gas from the ozone generator was bubbled into two aliquots of CH_2Cl_2 (100 mL each) for 30 sec and 60 sec respectively. The containers of these solutions were capped, and the absorbance of the two samples was measured at 260 nm within 30 minutes of the exposure to the ozone generator gas. The containers were capped, sealed with Parafilm, and stored in the -20º C freezer for 4 days; and the absorbance was again measured.

Observations:

1. The 60 second sample was almost two times more concentrated than the 30 second sample (based on initial absorbance readings).
2. The absorbance dropped precipitously when the cuvette was left uncapped, indicating rapid loss of ozone to the atmosphere. The absorbance reading of the 60 second sample dropped from 3.5 to 1.5 in one minute.
3. The absorbance reading stabilized after a cap was added. The absorbance reading even stabilized for about 30 sec. at ~1.1 absorbance units after the addition of a cap.
4. Most of the ozone had either diffused out of the containers or broken down after 4 days. The 30 sec sample had an absorbance of 0.036, while the 60 sec sample had an absorbance of 0.037 (compared to initial absorbances of 1.8 and 3.5 respectively).

These observations suggest that the only way of reproducibly adding ozone is to measure the absorbance of an ozone solution that is not highly saturated before transferring aliquots of ozone solution to the lycopene solution. This new approach has not yet been tried in the ozonolysis of lycopene.

Conclusion

This project has successfully synthesized or isolated the following products: β-cyclocitral (**1**), β-cyclogeranic acid (**2**), β-ionone (**3**), β-ionylideneacetaldehyde (**4**), β-ionylidene acetic acid (**5**), β-apo-13-carotenone (**6**), β-apo-14'-carotenal (**7**), β-apo-14'-carotenoic acid (**8**), β-apo-8'-carotenal (**11**), β-apo-8'-carotenoic acid (**12**). All synthesized and isolated compounds have been submitted for biological testing and will be tested in a variety of biological contexts in the future in the laboratory of Dr. Earl Harrison (Department of Nutrition).

The first tested hypothesis was that these eccentric cleavage products activate retinoic acid receptors (RARs). This hypothesis was tested by observing the effects of apo-carotenoids on the activation of RARα and RARβ transfected into monkey kidney cells along with retinoic acid response element-luciferase reporter constructs. The results for the activation of RARα are summarized in Figure 10. The apo-carotenoids have not been shown to activate retinoic acid receptors. However, some of these molecules appear to be antagonists to the action of retinoic acid on RARs in more recent testing.

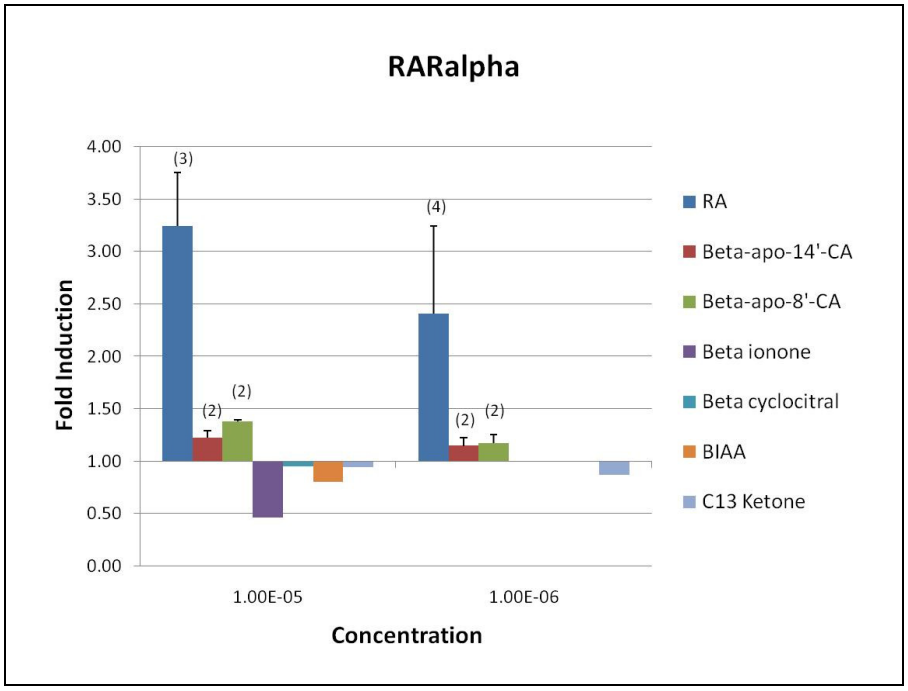

Figure 10. Effect of apo-carotenoids on the fold induction of RARα compared to the effect of retinoic acid.

β-apo-12'-carotenal (**9**), β-apo-12'-carotenoic acid (**10**), β-apo-10'-carotenal (**13**), and β-apo-10'-carotenoic acid (**14**) have yet to be synthesized and characterized. The synthesis of β-apo-14'-carotenal needs to be optimized so that it can be used as a starting material for these longer chain apo-carotenoids.

Our initial attempts at lycopene ozonolysis have been shown to produce desired apo-lycopenals. Further investigation is needed to determine the ideal degree of ozonolysis for production of putative lycopenal metabolites.

Experimental Section

General Methods

Sigma–Aldrich (Milwaukee, WI) supplied starting materials, reagents, and NMR solvents. All reactions and handling of retinoid-containing compounds were done under gold fluorescent lights. TLC was performed on EMD (Darmstadt, Germany) silica gel 60 F_{254} aluminum plates. Column chromatography was performed with Merck silica gel 60, 70-230 and 230-400 mesh (Sigma-Aldrich), and reverse-phase flash chromatography with Merck Lichroprep® RP-18. Preparative TLC was performed with Analtech (Newark, DE) 20 × 20 cm, 1000 micron Silica Gel GF plates with UV 254 indicator or Analtech 20 × 20 cm, 1000 micron reverse-phase hydrocarbon-impregnated silica plates with UV 254 indicator. Analytical HPLC was done on a Beckman Instruments (San Ramon, CA) unit, with model 127 pump and detector module 166, unless otherwise noted, equipped with a Metachem Polaris (Varian), 5 μm C-18, 250 × 4.6 mm column. Ultraviolet spectra were recorded on a Beckman Instruments DU-40 spectrophotometer. NMR spectra were recorded either on a Bruker (Billerica, MA) DRX 400 spectrometer or a Bruker Avance 300 spectrometer. Mass spectra were recorded on a Micromass (Milford, MA) QTOF Electrospray (ES) mass spectrometer in the Ohio State Chemical Instrumentation Center. Ozone generation from oxygen (at 8 psi) was performed using a Welsbach Ozonator (Philadelphia, PA) set at 105 volts. All reactions were performed using oven-dried glassware and run under a dry argon atmosphere, unless otherwise noted. Solutions were concentrated under vacuum using a rotary evaporator. All TLC analysis was done a 4:1 hexanes:ethyl acetate solvent system.

*β-Cyclocitral (Compound **1**)*

Crude purchased product was purified through preparative TLC using 9:1 hexanes:ethyl acetate. The desired band was removed and extraction with dichloromethane yielded the product. ^{1}H-NMR ($CDCl_3$) δ 1.23 (6H, s), 1.46-1.49 (2H, m), 1.63-1.1.71 (2H, m), 2.13 (3H, s), 2.21-2.24 (2H, m) 8.10 (1H, s); UV-Vis λ = 230 nm, ε = 2760.

*β-Cyclogeranic acid (Compound **2**)*

Purified **1** was dissolved in dichloromethane and left to stir for several days in solution exposed to the oxygen in the atmosphere. Preparative TLC purification yielded **2**. ^{1}H-NMR (DMK-D6) δ 1.14 (6H, s), 1.45-1.49 (2H, m), 1.66-1.71 (2H, m), 2.01-2.04 (2H, m), 2.08 (3H, m); UV-Vis λ = 230 nm, ε = 7720.

*β-Ionone (Compound **3**)*

Column chromatography of crude **3** in 17:3 hexanes:ethyl acetate yielded purified **3**. ^{1}H-NMR ($CDCl_3$) δ 1.05 (6H, s), 1.44-1.47 (2H, m), 1.57-1.62 (2H, m), 1.74 (3H, s), 2.03-2.06 (2H, m), 2.27 (3H, m), 6.06 (1H, d, *J* = 16.0 Hz), 7.25 (1H, d, *J* = 16.4 Hz); ^{13}C-NMR ($CDCl_3$) δ 18.67, 21.50, 26.93, 28.57, 33.32, 33.84, 39.52, 131.35, 135.71, 135.78, 142.89, 198.38; HRMS (ES) calculated for $C_{13}H_{20}O$ (M+H): 193.1592. Found: 193.1579.

*β-Ionylidene acetic acid ethyl ester (ethyl ester of **5**)*

Triethylphosphonoacetate (1.94 mL; 9.67 mmol) and sodium hydride (290 mg; 12.1 mmol) were dissolved in dry THF (25 mL). This solution stirred for 0.5 h after visible bubbling ceased. β-ionone (1.24 g; 6.45 mmol) was dissolved in dry THF (25 mL) and added dropwise to the

reaction mixture. The reaction stirred under reflux for 4 h and was left to stir overnight at rt. The reaction was worked up through a standard ethyl acetate-water extraction (The reaction mixture, ~50 mL of ethyl acetate, and ~30 mL of distilled water were shaken in a separatory funnel. The aqueous layer was separated after equilibration. The organic layer was washed with brine and isolated. The aqueous layer was again extracted with ~25 mL of ethyl acetate. The organic layer was washed with brine and combined with the earlier organic layer. The combined organic layers were dried with anhydrous sodium sulfate and vacuum filtered through sintered glass). The ethyl acetate extract was isolated and concentrated. Purification through column chromatography (silica gel, 9:1 hexanes:ethyl acetate) yielded the product (1.02 g; 3.87 mmol, 93.6% yield) and β-ionone (0.44 g; 2.3 mmol). ^{1}H-NMR ($CDCl_3$) δ 1.06 (6H, s), 1.30 (3H, t, *J* = 7.2 Hz), 1.49-1.52 (2H, m), 1.63-1.67 (2H, m), 1.73 (3H, s), 2.04-2.08 (2H, m), 2.37 (3H, m), 4.21 (2H, q, *J* = 7.1 Hz), 5.78 (1H, s), 6.13 (1H, d, *J* = 16.1 Hz), 6.59 (1H, d, *J* = 16.0 Hz).

*β-Ionylideneacetaldehyde (Compound **4**)*
LAH (120 mg; 3.2 mmol) was dissolved in dry THF (10 mL). β-ionylidene acetic acid ethyl ester (210 mg; 0.81 mmol) was dissolved in dry THF and added dropwise to the LAH solution. The reaction stirred for 45 min. and 120 μL H_2O, 120 μL 15% NaOH, and 3 x 120 μL H_2O were added to the solution. The solution was vacuum filtered and treated with an ethyl acetate-water extraction. The ethyl acetate extract was concentrated. The crude alcohol product was passed through a column containing MnO_2 (1.7 g; 20 mmol) and diatomaceous earth (18 g) suspended in dichloromethane. Elution lasted 4 h. Column chromatography (silica gel, 9:1 hexanes:ethyl acetate) yielded the product (140 mg; 0.59 mmol, 73% yield). ^{1}H-NMR ($CDCl_3$) δ 1.05 (6H, s), 1.46-1.55 (2H, m), 1.60-1.67 (2H, m), 1.74 (3H, s), 2.03-2.10 (2H, m), 2.39 (3H, s), 5.96 (1H, d, *J* = 8 Hz), 6.23 (1H, d, *J* = 16 Hz), 6.74 (1H, d, *J* = 16 Hz), 10.14 (1H, d, *J* = 8 Hz); HRMS (ES) calculated for $C_{15}H_{22}O$ (M+H): 219.1749. Found: 219.1737.

*β-Ionylidene acetic acid (Compound **5**)*
β-Ionylidene acetic acid ethyl ester (93 mg, 0.36 mmol) was dissolved in ethanol (5 mL). Aqueous 5 N KOH (0.71 mL; 3.6 mmol) was added to the solution. The reaction stirred overnight. TLC analysis indicated full conversion to the acid. The solution was concentrated, acidified using 6 N HCl, and immediately extracted with ethyl acetate. The aqueous layer was extracted twice with ethyl acetate. The ethyl acetate layers were concentrated to yield the product (83 mg; 0.35 mmol, 99% yield). ^{1}H-NMR ($CDCl_3$) δ 1.07 (6H, s), 1.50-1.53 (2H, m), 1.63-1.69 (2H, m), 1.75 (3H, s), 2.06-2.09 (2H, m), 2.36 (3H, s), 5.85 (1H, s), 6.23 (1H, d, *J* = 16.2), 6.67 (1H, *J* = 16.2); UV-Vis λ_{max} = 341 nm, ε = 20,000; HRMS (ES) calculated for $C_{15}H_{22}O_2$ (M+H): 235.1698. Found: 235.1688.

*β-Apo-13-carotenone (Compound **6**)*
1-Triphenylphosphoranylidene-2-propanone (330 mg; 1.0 mmol) and β-ionylideneacetaldehyde (130 mg, 0.59 mmol) were dissolved in dry toluene (20 mL). The solution was refluxed overnight. Evaporation of solvent and preparative TLC (silica gel, 17:3 hexanes:ethyl acetate) yielded the product (90 mg; 0.36 mmol, 61% yield). ^{1}H-NMR ($CDCl_3$) δ 0.98 (6H, s), 1.41-1.44 (2H, m), 1.54-1.60 (2H, m), 1.67 (3H, s), 1.99-2.00 (2H, m), 2.01 (3H, m), 2.25 (3H, s), 6.08-6.15 (3H, m), 6.37 (1H, d, J = 16.0 Hz), 7.53 (1H, dd, J = 15.0, 11.9 Hz); ^{13}C-NMR ($CDCl_3$) δ 13.10, 19.14, 21.72, 27.63, 28.93, 33.14, 34.25, 39.59, 76.58, 77.01, 77.20, 77.43, 127.67,

129.27, 130.98, 131.27, 136.68, 137.48, 139.30, 145.55, 198.48; UV-Vis λ_{max} = 341 nm; HRMS (ES) calculated for $C_{18}H_{26}O$ (M+Na): 281.1881. Found: 281.1859.

*β-Apo-14'-carotenal (Compound **7**)*
(Triphenylphosphoranylidene)acetaldehyde (1.21 g; 4.0 mmol) and retinal (580 mg; 2.0 mmol) were dissolved in dry toluene (30 mL). The mixture was refluxed for two days. The reaction mixture was concentrated and suspended in ethyl ether. This was stored in the freezer overnight. The suspension was vacuum filtered. Column chromatography (initially in 19:1 hexanes:ethyl acetate, then in 9:1 hexanes:ethyl acetate) yielded a crude product that matched the rf of **7**. TLC analysis indicated that **7** shares the same rf as retinal on silica gel. RPTLC analysis indicated that retinal was present in the product mixture. Reverse phase column chromatography (9:1 methanol:water) yielded purified **7** (25 mg, 4% yield). ^{1}H-NMR ($CDCl_3$) δ 1.00 (6H, s), 1.40-1.44 (2H, m), 1.54-1.60 (2H, m), 1.70 (3H, s), 1.93-2.04 (2H, m), 2.00 (3H, s), 2.07 (3H, s), 6.10-6.44 (6H, m), 6.90 (1H, dd, *J* = 14.9, 11.5 Hz), 7.50 (1H, dd, *J* = 12.0, 14.9 Hz), 9.58 (1H, d, *J* = 7.9).

*Ethyl β-apo-14'-carotenoate (ethyl ester of compound **8**)*
Triethylphosphonoacetate (2.52 mL; 12.6 mmol) and sodium hydride (350 mg; 14.6 mmol) were dissolved in dry THF (25 mL). This solution stirred for 1.5 h after visible bubbling ceased. Retinal (1.20 g; 4.20 mmol) was dissolved in dry THF (25 mL) and added dropwise to the reaction mixture. The reaction was stirred for 48 h. The reaction was worked up through a standard ethyl acetate-water extraction. The ethyl acetate extract was concentrated. Purification through column chromatography (silica gel, 9:1 hexanes:ethyl acetate) yielded the product (1.10 g; 3.12 mmol, 74.2% yield). ^{1}H-NMR ($CDCl_3$) δ 1.00 (6H, s), 1.28 (3H, t, *J* = 7.2), 1.42-1.53 (2H, m), 1.52-1.57 (2H, m), 1.69 (3H, s), 1.96 (3H, s), 1.96-2.04 (2H, m), 2.04 (3H, s), 4.19 (2H, q, *J* = 7.2), 5.86 (1H, d, J = 15.0 Hz), 6.10-6.25 (4H, m), 6.32 (1H, d, *J* = 15.1 Hz), 6.81 (1H, dd, *J* = 11.5, 15.0 Hz), 7.68 (1H, dd, *J* = 15.1, 11.9).

*β-apo-14'-carotenoic acid (Compound **8**)*
Ethyl β-apo-14'-carotenoate (120 mg, 0.34 mmol) was dissolved in ethanol (5 mL). Aqueous 5 N KOH (0.68 mL; 3.4 mmol) was added to the solution. The reaction stirred overnight. TLC analysis indicated full conversion to the acid. The solution was concentrated, acidified using 6 N HCl, and immediately extracted with ethyl acetate. The aqueous layer was extracted twice with ethyl acetate. The ethyl acetate layers were concentrated to yield the product (104 mg; 0.32 mmol, 94% yield). ^{1}H-NMR ($CDCl_3$) δ 1.05 (6H, s), 1.46-1.54 (2H, m), 1.61-1.71 (2H, m), 1.77 (3H, s), 2.06 (3H, s), 2.06-2.10 (2H, m), 2.13 (3H, s), 5.92 (1H, d, J = 15.0 Hz), 6.13-6.32 (4H, m), 6.39 (1H, d, *J* = 15.1 Hz), 6.90 (1H, dd, *J* = 11.5, 15.0 Hz), 7.88 (1H, dd, *J* = 15.1, 11.9).

*β-apo-8'-carotenoate (Compound **11**)*
Purchased from manufacturer. ^{1}H-NMR ($CDCl_3$) δ 1.07 (6H, s), 1.49-1.52 (2H, m), 1.60 (3H, s), 1.60-1.69 (2H, m), 1.76 (3H, s), 1.94 (3H, s), 2.02-2.07 (2H, m), 2.02 (3H, s), 2.04 (3H, s), 6.15-6.23 (3H, m), 6.31 (1H, d, *J* = 11.6 Hz), 6.40 (1H, d, *J* = 14.8 Hz), 6.49 (1H, d, *J* = 11.6 Hz), 6.64-6.84 (5H, m), 6.98 (1H, d, *J* = 10.7 Hz), 9.49 (1H, s).

*Methyl β-apo-8'-carotenoate (methyl ester of compound **12**)*
β-apo-8'-carotenal (Compound **11**, 500 mg; 1.2 mmol), KCN (550 mg; 8.4 mmol), MnO_2 (2.5 g; 29 mmol), and acetic acid (150 μL; 29 μmol) were suspended in methanol (50 mL) and stirred at rt for 90 h. The suspension was filtered. The methanol solution was concentrated and extracted between aqueous sodium bicarbonate and ethyl acetate. The ethyl acetate extract was concentrated. Column chromatography (silica gel, 19:1 hexanes:ethyl acetate) and subsequent preparative TLC (silica gel, 9:1 hexanes:ethyl acetate) yielded the product (11 mg; 2% yield). ^{1}H-NMR ($CDCl_3$) δ 1.07 (6H, s), 1.47-1.52 (2H, m), 1.59 (3H, s), 1.64-1.67 (2H, s), 1.76 (3H, s), 2.02 (3H, s), 2.03 (3H, s), 2.06 (3H, s), 2.02-2.06 (2H, m), 3.81 (3H, s), 6.18-6.31 (4H, m), 6.37-6.41 (2H, m) 6.55-6.76 (5H, m), 7.33 (1H, d, J = 11.2).

Retinal (from retinyl acetate)
Retinyl acetate (1.06 g; 3.23 mmol) was dissolved in ethanol (40 mL). Aqueous KOH (5 N, 10 mL) was added to the solution. The reaction stirred under argon for 5.5 h. The volume of the solution was reduced, acidified with aqueous 6N HCl and extracted twice with ethyl acetate. The ethyl acetate extract was concentrated and purified through flash chromatography (silica gel, 3:1 hexanes:ethyl acetate) to yield retinol (0.56 g; 64% yield). Retinol (1.40 g; 4.90 mmol) was dissolved in CH_2Cl_2 and eluted through a column containing MnO_2 (8.57 g; 98.6 mmol) and diatomaceous earth (43 g) suspended in CH_2Cl_2. A 7 h elution yielded crude retinal (1.14 g; 81.2% yield).

Tomato oleo resin extraction
Tomato paste (12 oz.), methanol (600 mL), diatomaceous earth (100 g), and calcium carbonate (70 g) were mixed in a blender for 5 min. The methanol was separated through vacuum filtration and discarded. The filtrate was mixed in a blender with acetone (600 mL), and the acetone was separated through vacuum filtration and discarded. The filtrate was then mixed with 1:1 acetone:hexanes (600 mL). The acetone/hexane solution was separated through vacuum filtration and kept. The filtrate was again extracted with 1:1 acetone:hexane (600 mL), and the filtrate was combined with the other acetone/hexane filtrate. The combined filtrates were washed with brine (3 × 100 mL), and the organic layer was concentrated to leave a crude tomato oleo resin.

Crude lycopene isolation
Oleo resin isolated from a 12 oz can of tomato paste was dissolved in CH_2Cl_2 in an rb flask, and RP C-18 silica gel was added. The CH_2Cl_2 was evaporated, and the reverse phase gel was transferred to a reverse-phase column in a 45:45:10 MeOH:CH_3CN:CH_2Cl_2 mobile phase. The column was washed with 150 mL of mobile phase, and then pure CH_2Cl_2 was added to elute the lycopene .

Lycopene ozonolysis
Crude lycopene (4 mg; 0.01 mmol) and 4-methylmorpholine-N-oxide (40 mg; 0.34 mmol) were dissolved in CH_2Cl_2 (8 mL). This solution was separated into four 2 mL. Phosphate buffer (25 mM, pH = 6; 2 mL) was added to each solution. Gas from the ozone generator was bubbled into a separate vial of CH_2Cl_2 for 30 seconds. Different aliquots (0.5 mL, 1 mL, 1.5 mL, and 2 mL) of the ozone-saturated CH_2Cl_2 were transferred to one each of the four vials. Additional gas from the ozone generator was bubbled through the ozone-saturated CH_2Cl_2 for 10 seconds before

every subsequent transfer after the first transfer. The vials were shaken immediately upon ozone addition to quench with the phosphate buffer. The dichloromethane layers were then separated, concentrated, and stored.

Lycopene cleavage HPLC analysis

Ozonolysis products were analyzed using a gradient from 75:25 MeOH:water to 100% MeOH over 25 minutes. Both the methanol and the water fractions of the mobile phase were buffered with ammonium acetate (10 mM).

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Appendix

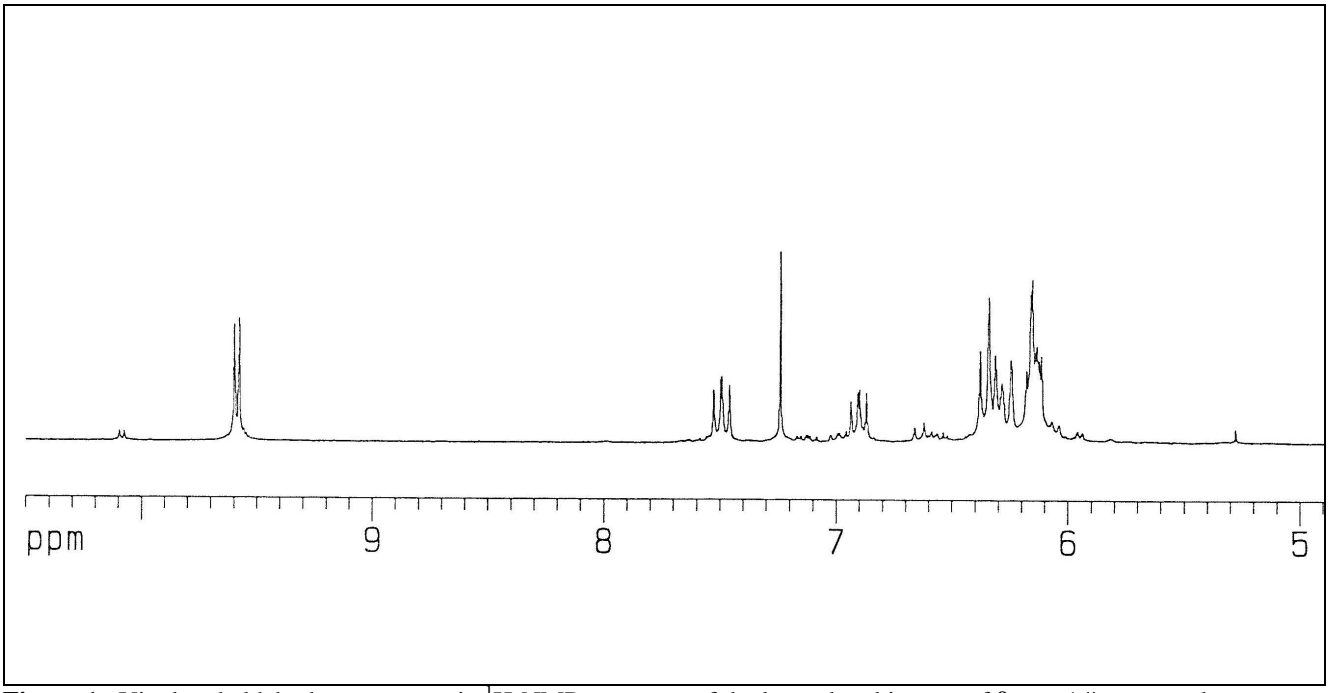

Figure 1. Vinyl and aldehyde resonances in ^{1}H-NMR spectrum of the lower band isomer of β-apo-14`-carotenal.

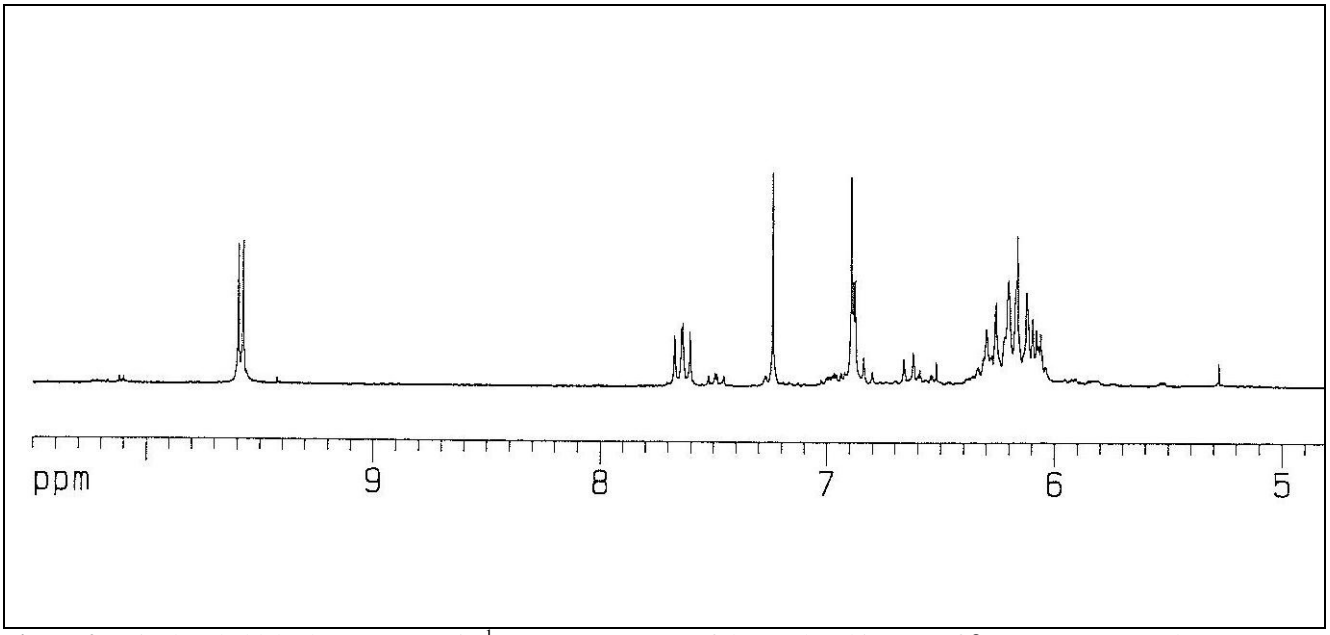

Figure 2. Vinyl and aldehyde resonances in ^{1}H-NMR spectrum of the top band isomer of β-apo-14`-carotenal.

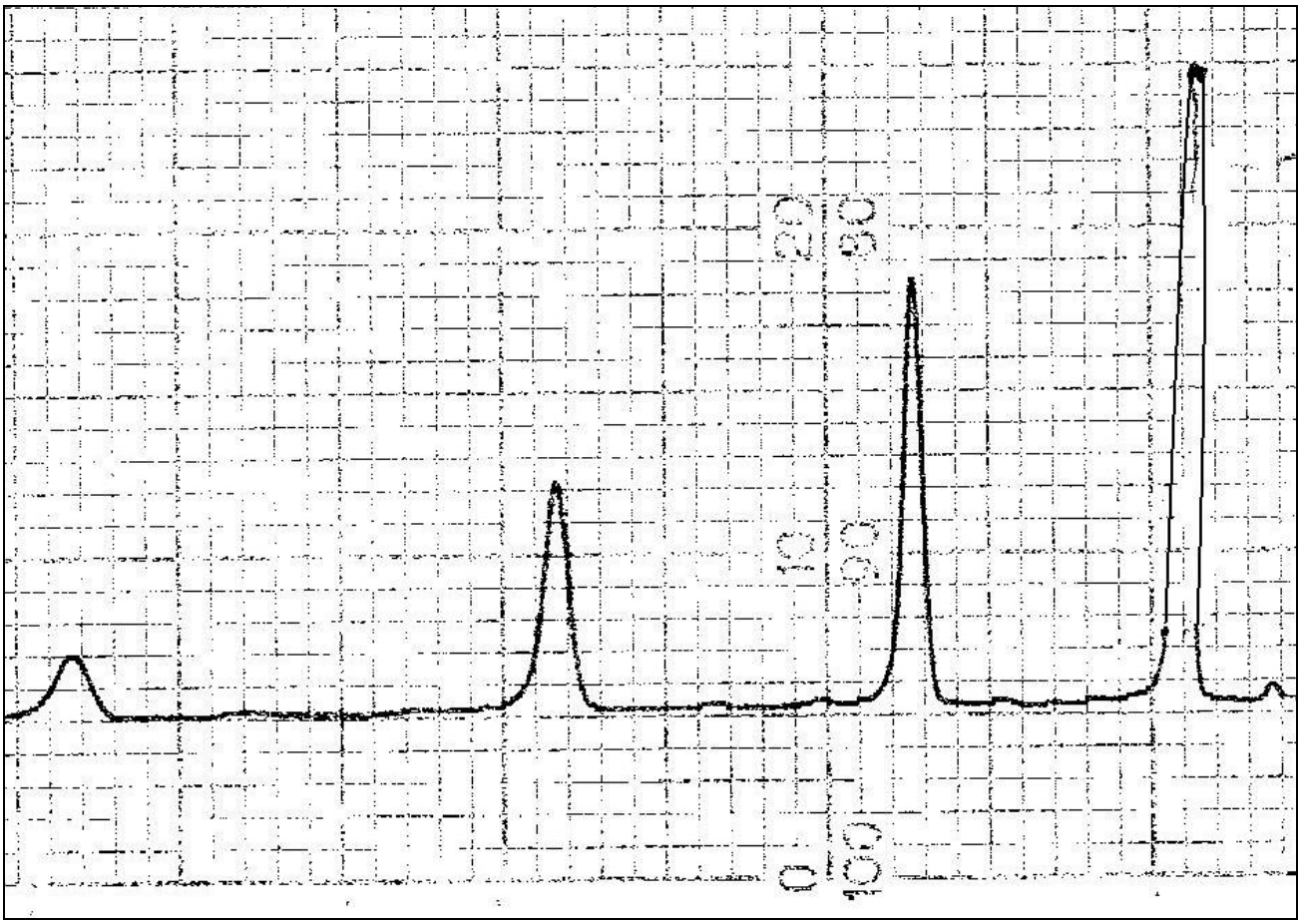

Figure 3. Portion of a chromatogram after initial silica gel purification of the Wittig synthesis of β-apo-14`-carotenal. This indicates the need for an additional reverse-phase purification step.

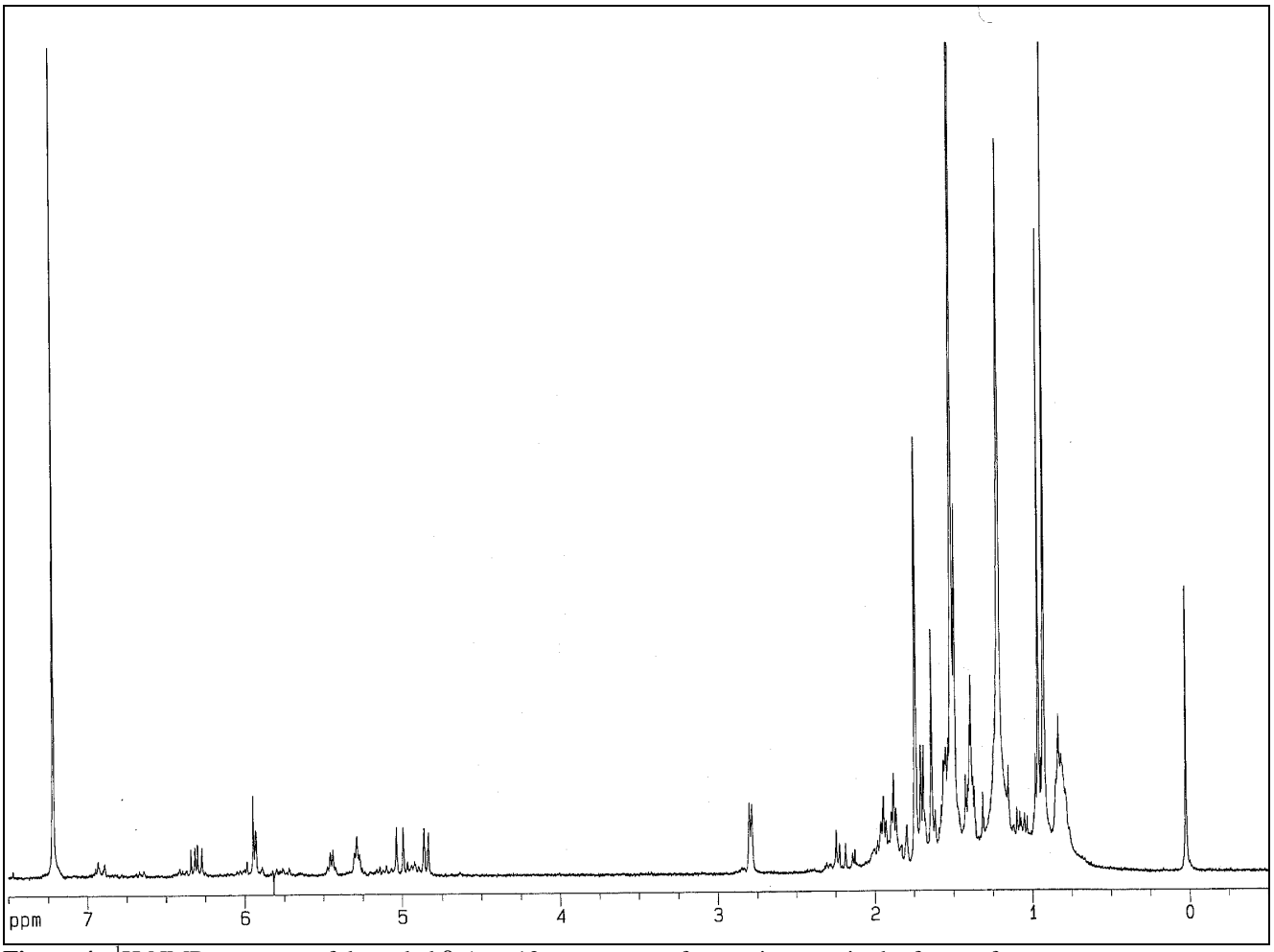

Figure 4. ^{1}H-NMR spectrum of degraded β-Apo-13-carotenone after storing neat in the freezer for a year.

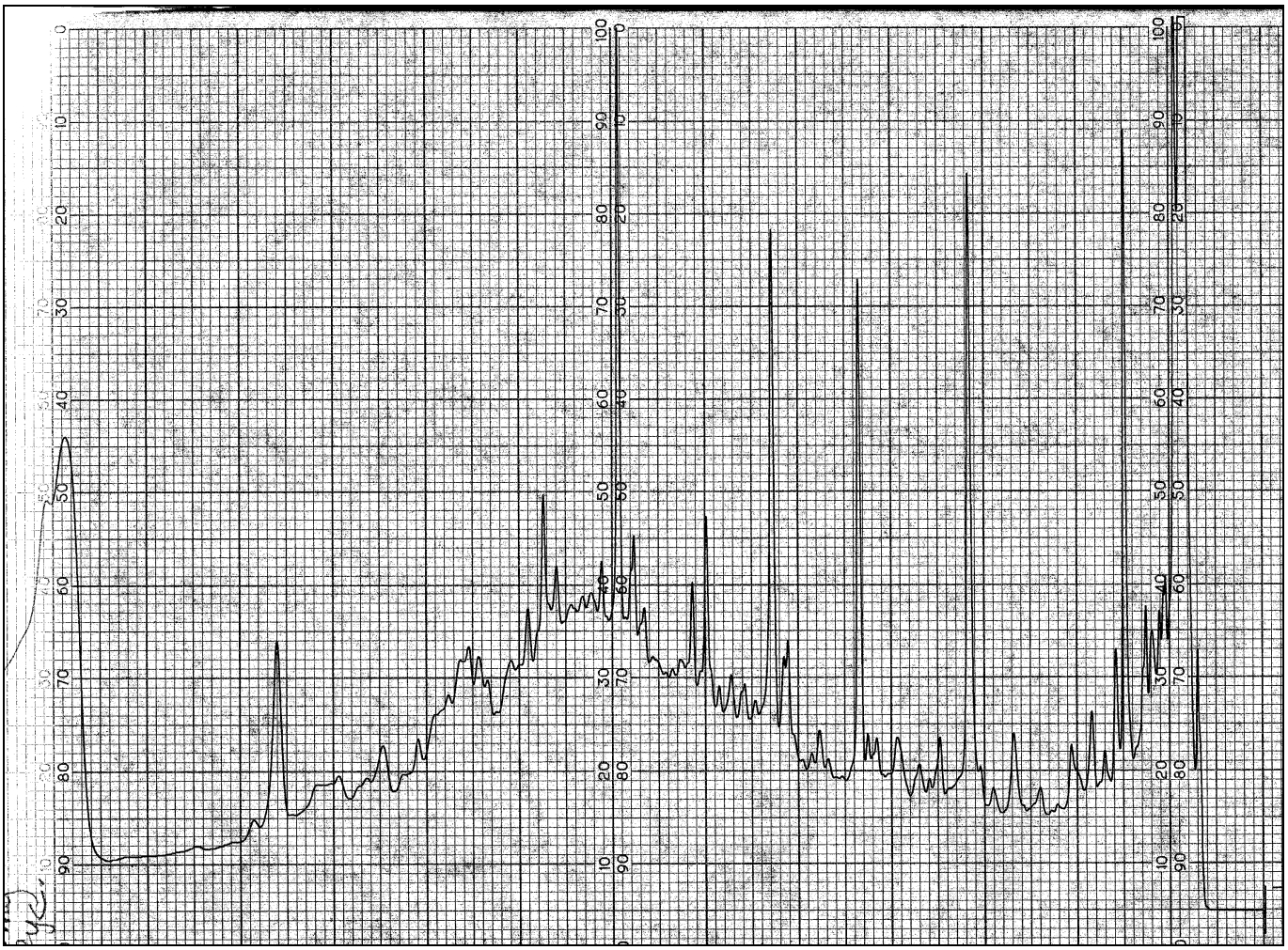

Figure 5. Portion of chromatogram after a lycopene ozonolysis.